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Squamous cell carcinoma antigen 1 is an inhibitor of parasite-derived cysteine proteases

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Abstract **The physiological significance of the squamous cell carcinoma antigens 1 (SCCA1) and SCCA2, members of the ovalbumin serpin family, remains unresolved. In this study, we examined whether SCCA1 or SCCA2 inhibits protozoa- or helminth-derived cysteine proteases. SCCA1, but not SCCA2, potently inhibited the cysteine protease activities of CPB2.8 from *Leishmania mexicana*, cruzain from *Trypanosoma cruzi*, rhodesain from *Trypanosoma brucei rhodesience*, and cathepsin L2 from *Fasciola hepatica*. The inhibitory activities of SCCA1 were due to its resistance to cleavage by the cysteine proteases. The findings indicate that induction of cysteine protease inhibitors might be a novel defense mechanism against parasite development.**

Keywords: Serpin, Interleukin-4; Interleukin-13; Cysteine protease; Parasite; Inhibitor

1. Introduction

The squamous cell carcinoma antigens 1 (SCCA1) and SCCA2 belong to the ovalbumin-serpin family [1]. Although SCCA1 and SCCA2 are closely homologous (91% identical at the amino acid level), these two molecules have distinct properties: SCCA1 inhibits cysteine proteases such as cathepsin K, L, and S, whereas SCCA2 inhibits serine proteinases such as cathepsin G and human mast cell chymase [1]. The distinct specificities of SCCA1 and SCCA2 are due to the differences in their reactive site loop (RSL) sequences, with only 7 amino acid residues among 13 in the RSL (amino acid 347–359) shared between them. We have previously shown that both SCCA1 and SCCA2 are induced by two related Th2-type cytokines, interleukin (IL)-4 and IL-13, in bronchial epithelial cells and keratinocytes [2,3], and that SCCA2, but not SCCA1, inhibited the cysteine protease activities of a major mite allergen, Der p 1 [4], indicating that SCCA proteins act as a defense mechanism against extrinsic proteases. However, the physiological significance of SCCA molecules remains unresolved.

Many protozoan and helminth parasites generate abundant Clan CA, Family C1 cysteine proteases, which are orthologues of mammalian cathepsin L [5]. Cysteine proteases in parasitic protozoa and helminths play key functions in differentiation of the parasites themselves, infection to the hosts, and immunomodulation of the hosts [5]. In contrast, Th2-type immune responses, particularly IL-4 and IL-13, act as a defense mechanism against parasites, mainly helminths [6]. While activation of mast cells and eosinophils in Th2-type immune response is critical for expulsion of parasites, other non-immune mechanisms, such as mucus production, smooth muscle hyperresponsiveness, increased intestinal permeability, and fibrosis, are also important [6]. However, thus far, it is unknown whether protease/protease inhibitor interaction is involved in the defense mechanism against parasites.

In this study, we explored the possibility that SCCA1 and/or SCCA2 might inhibit the catalytic activities of parasite-derived cysteine proteases, thereby acting as a possible defense mechanism against invading parasites.

2. Materials and methods

2.1. Plasmids and generation of recombinant proteins

Plasmids encoding wild type SCCA1, SCCA2, and mutant types of SCCA were prepared as previously described [4,7,8]. Glutathione *S* transferase-fused SCCA proteins and recombinant proteins of CPB2.8 from *L. mexicana*, cruzain from *T. cruzi*, rhodesain from *T. brucei rhodesience*, and cathepsin L1 (FheCL1) and FheCL2 from *F. hepatica* were generated as previously described [4,7–12].

2.2. Cells and transfection

Normal human epidermal keratinocytes (neonatal skin) were cultured according to the manufacturer's procedure (Clonetics). For stimulation, cells were cultured with 10 ng/ml of human IL-4 or 50 ng/ml of human IL-13 (Peprotech). HEK293T cells were cultured

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Abbreviations: IL, interleukin; SCCA, squamous cell carcinoma antigen; RSL, reactive site loop; *Fasciola hepatica* cathepsin L, FheCL; *Endo* F, *N*-glycosidase F; MALDI-TOF, matrix-associated laser desorption ionization time-of-flight; Ab, antibody; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

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and transfection of the plasmids was performed as previously described [8]. In some experiments, 82 h after IL-4/IL-13 was added, cells were incubated with 2 μg/ml brefeldin A (Sigma) for 14 h at 37 °C, and the cell lysates were incubated with 25 U/μl of *N*-Glycosidase F (Endo F, New England Biolabs) for 12 h at 37 °C.

2.3. Enzyme assays

Enzyme assays of cysteine proteases were performed modifying the previous methods [4,7,8]. The enzymes were pre-activated by incubation with 10 mM DTT for 10 min at 37 °C. After incubation of either 0.5 nM (CPB2.8, cruzain or rhodesain) or 5 nM (FheCL1 or FheCL2) of cysteine proteases with the reaction buffer (400 mM sodium acetate; pH 5.0, 4 mM EDTA, 8 mM DTT, and 0.001% BSA) for 10 min at 37 °C, the indicated concentration of GST-fused SCCA proteins was added to the mixture followed by incubation for 20 min at 37 °C. Upon addition of 25 μM substrate to the reaction mixture, the residual enzyme activity was measured.

2.4. Cleavage assay of SCCA proteins by cysteine proteases

Two micromolar of pre-activated cysteine proteases and SCCA proteins were mixed in phosphate reaction buffer for various time periods at 37 °C or for 2 h at 4 °C. Then the reactive samples were subjected to SDS–PAGE or Voyager RP matrix-associated laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (PerSeptive Biosystems).

2.5. Western blotting

Western blotting was carried out as previously described [4,7,8]. The membranes were probed with 100-fold diluted anti-SCCA1 or anti-GAPDH antibody (Ab, Santa Cruz Biotechnology).

2.6. Amino acid sequencing analysis

The peptides transferred to PVDF membranes (BIO-RAD) and stained with Coomassie Brilliant Blue R250 in 50% methanol were subjected to an Applied Biosystems Procise 494 HT protein sequencer (ABI Applied Biosystems).

3. Results

3.1. Inhibition of catalytic activities of parasite-derived cysteine proteases by SCCA 1

To examine whether SCCA proteins inhibits the catalytic activities of parasite-derived cysteine proteases, we chose CPB2.8 from *L. mexicana*, cruzain from *T. cruzi*, and rhodesain from *T. brucei rhodesience*, as cysteine proteases of protozoa, and FheCL1 and FheCL2 from *F. hepatica*, as cysteine proteases of helminths. The whole identities between human cathepsin L and these proteases are 28–41%, and the identities among these parasite proteases are 25–78%. FheCL1 and FheCL2 share the highest homology, 78% (Suppl. Fig. 1). The catalytic triads formed by cysteine, histidine, and asparagine residues are conserved in all these proteases.

SCCA1 potently inhibited the catalytic activities of CPB2.8, cruzain, rhodesain, and FheCL2, in a dose-dependent manner, but was less effective against FheCL1, whereas SCCA2 did not show such potency (Fig. 1A–E). The inhibitory activities of SCCA1 against parasite-derived cysteine proteases were almost equal to those against human cathepsin L and papain [7]. In contrast, neither SCCA1 nor SCCA2 inhibited Arg- and Lys-gingipains derived from *Porphyromonas gingivalis*, major virulence factors in pathogenesis of periodontitis (data not shown, Ref. [13]), showing the existence of the specificity

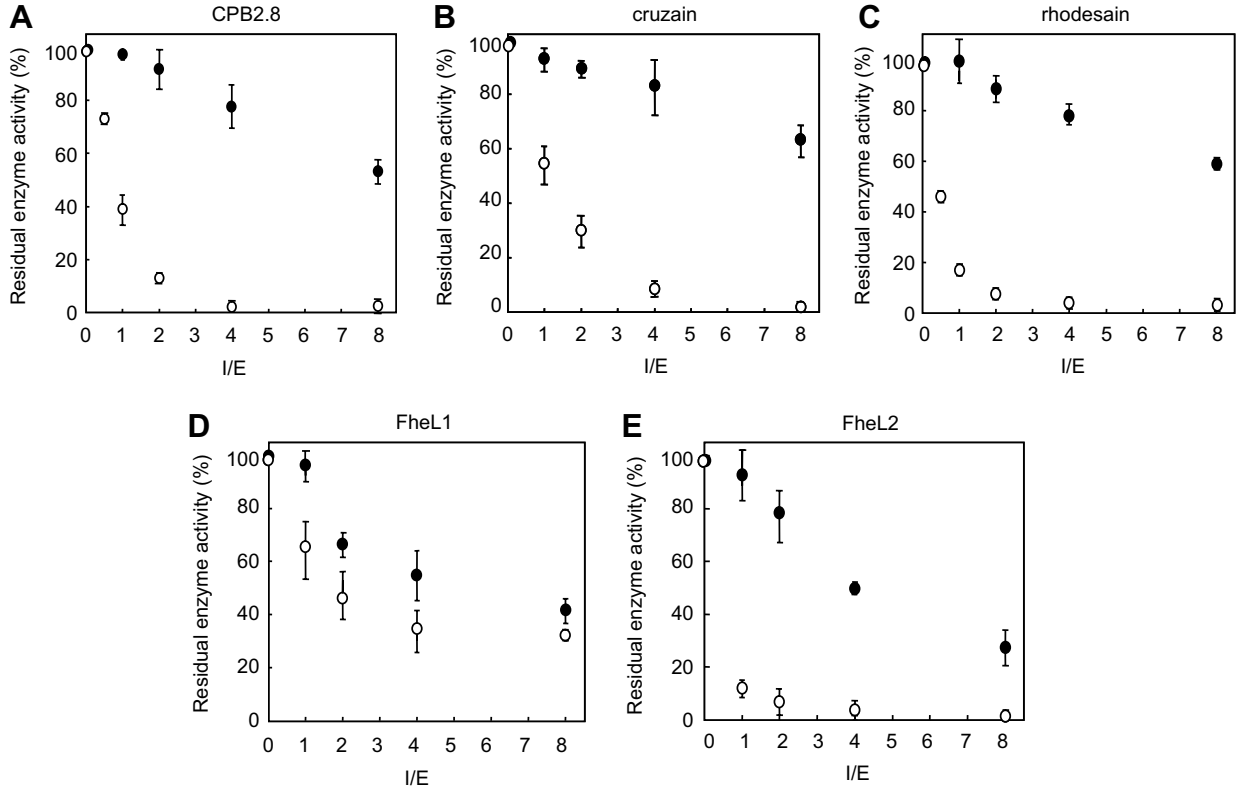

Fig. 1. Inhibition of parasite-derived cysteine proteases by SCCA proteins. CPB2.8 (A; 0.5 nM), cruzain (B; 0.5 nM), rhodesain (C; 0.5 nM), FheCL1 (D; 5 nM), or FheCL2 (E; 5 nM) was incubated with SCCA1 (open circles) or SCCA2 (closed circles) at the indicated inhibitor/enzyme (I/E) ratio. The residual enzyme activities are depicted. The same experiments were performed at least three times, and the mean values are shown.

of SCCA1 for microbe-derived cysteine proteases. These results demonstrated that SCCA1 had broad-spectrum inhibitory activity on a variety of parasite-derived cathepsin L-like cysteine proteases.

3.2. Resistance of SCCA1 to cleavage by parasite-derived cysteine proteases

Serpins exert their protease-inhibitory activities by the suicide substrate-like mechanism, in which the RSLs of serpins are recognized by proteases as a bait mimicking a normal substrate [1]. We have previously demonstrated, however, that the RSL of SCCA2 inhibited Der p 1 by binding to its active site but resisting cleavage [4]. Here, we investigated whether SCCA1 employs the same inhibitory mechanism on parasite-derived cysteine proteases. By incubation of SCCA1 with CPB2.8, cruzain, rhodesain and FheCL2 for 30 min, 42–75% of SCCA1 still existed, intact, although FheCL1 quickly degraded SCCA1 (Fig. 2A). In contrast, SCCA2 was immediately digested by all the investigated proteases except cruzain. The resistance of SCCA1 to cleavage by parasite-derived cysteine proteases was consistent with the inhibitory activities of SCCA1 against these proteases (see Fig. 1). Application of the cleaved peptides to MALDI-TOF mass spectrometry showed that the cleavage sites of CPB2.8, cruzain, rhodesain and FheCL2 within SCCA1 were the same, the bond between Gly-351 and Phe-352 (Fig. 2B). Taken together, resistance of SCCA1 to cleavage by parasite-derived cysteine proteases contributed to the inhibitory activities of SCCA1 on these proteases.

3.3. Identification of the amino acids in SCCA1 critical for their inhibitory activities against parasite-derived proteases

It seemed likely that the distinct inhibitory properties of SCCA1 and SCCA2 towards parasite-derived cysteine proteases are due to the differences in their RSL sequences. The exchange of the RSL, including distal and proximal hinge regions of SCCA1, for that of SCCA2 attenuated the inhibitory effects, whereas the opposite exchange acquired the inhibitory effects, demonstrating that the inhibitory effect on parasite-derived cysteine proteases was predominantly dependent on this region of SCCA1 (Table 1, SCCA1 RSL2, SCCA2 RSL1). We then generated five mutated types of SCCA1, in which each amino acid specific for the RSL of SCCA1 was exchanged with that corresponding to SCCA2, and analyzed their inhibitory effects on parasite-derived cysteine proteases. Replacing Phe-352 or Gly-353 with Val or Glu, respectively, resulted in attenuation of the inhibitory effects (Table 1, SCCA1 mut2, SCCA1 mut3), whereas the other three mutations inhibited the proteases as well as SCCA1. Taken together with the results that the bond between Gly-351 and Phe-352 was the cleavage site by parasite-derived cysteine proteases (Fig. 2B), we concluded that Phe-352 and Gly-353 at the positions of P1′ and P2′ were critical for SCCA1 to exert its inhibitory effects on parasite-derived cysteine proteases.

3.4. Secretion of SCCA proteins from epithelial cells

If SCCA1 inhibits the catalytic activities of parasite-derived cysteine proteases in their mammalian hosts acting as a defense mechanism against the parasites, SCCA1 could be secreted from its producing cells and interact with the proteases extracellularly. However, the amino acid sequences of SCCA1 and SCCA2 do not show the existence of signal sequences in these N termini, according to the SignalP 3.0 server. Therefore, we investigated the secretory mechanism of SCCA proteins. Upon stimulation of IL-4 or IL-13 on human keratinocytes at 48 h, SCCA was detected inside the cells, but only slightly in the supernatants. But after 96 h, SCCA was clearly observed in the supernatants (Fig. 3A, data not shown). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a representative of cytosolic proteins, was not detected in the supernatants, suggesting that the existence of SCCA proteins in the supernatants was not due to leakage from dead cells. Treatment of brefeldin A, a classical inhibitor of ER/Golgi-dependent protein transport, on IL-4-treated keratinocytes did not attenuate secretion of SCCA proteins (Fig. 3B). Treatment of the cell lysates of IL-4/IL-13-stimulated keratinocytes with *N*-glycosidase F that cleaves *N*-glycosylated sugar chains did not change the mobilities of the SCCA proteins (Fig. 3C), although SCCA1/2 have four potential *N*-glycosylation sites. Furthermore, the amino acid sequencing of the secreted SCCA proteins from HEK293T cells showed that their N termini were intact (data not shown). Taken together, these results suggested that SCCA proteins could be secreted from epithelial cells by an ER/Golgi-independent pathway.

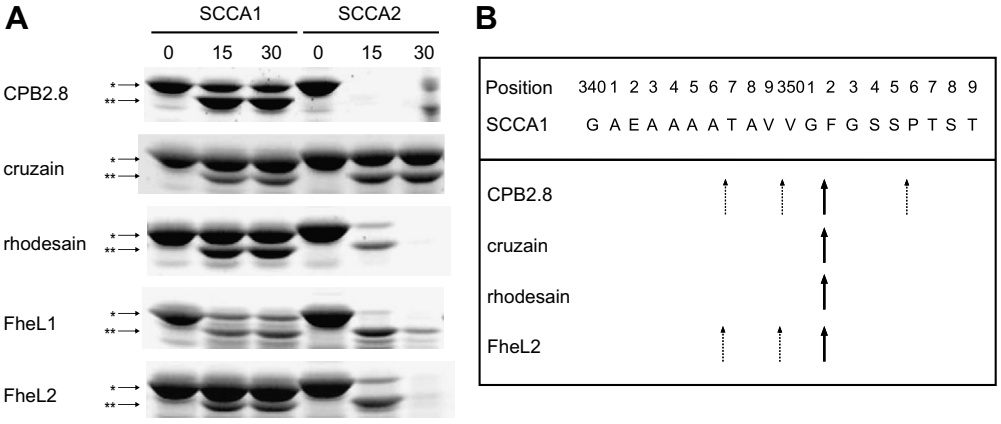

Fig. 2. Cleavage profiles and sites of SCCA1 by parasite-derived cysteine proteases. SCCA1 or SCCA2 proteins were incubated with each parasite-derived cysteine protease for the indicated times at 37 °C (A) or for 2 h at 4 °C (B) and subjected to SDS–PAGE followed by SYPRO Ruby staining (A) or MALDI-TOF mass spectrometry (B). In A, the single and double asterisks represent the intact and cleaved SCCA proteins, respectively. In B, the bold and dashed arrows represent the major and minor cleavage sites of SCCA1, respectively.

Table 1
Alignment of RSLs of SCCA proteins and their inhibitory activities

Position	Proximal hinge							Reactive site loop													Distal hinge
	340	1	2	3	4	5	6	7	8	9	350	1	2	3	4	5	6	7	8	9	364
SCCA1	G	A	E	A	A	A	A	T	A	V	V	G	F	G	S	S	P	T	S	T	H
SCCA2	•	V	•	•	•	•	•	•	•	•	•	V	V	E	L	•	S	P	•	•	C
SCCA1 RSL2	•	V	•	•	•	•	•	•	•	•	•	V	V	E	L	•	S	P	•	•	C
SCCA2 RSL1	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
SCCA1 mut1(G351V)	•	•	•	•	•	•	•	•	•	•	•	V	•	•	•	•	•	•	•	•	•
SCCA1 mut2(F352V)	•	•	•	•	•	•	•	•	•	•	•	•	V	•	•	•	•	•	•	•	•
SCCA1 mut3(G353E)	•	•	•	•	•	•	•	•	•	•	•	•	•	E	•	•	•	•	•	•	•
SCCA1 mut4(S354L)	•	•	•	•	•	•	•	•	•	•	•	•	•	•	L	•	•	•	•	•	•
SCCA1 mut5(P356S/ T357P)	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	S	P	•	•	•

• the same amino acid as SCCA1

	CPB2.8	Cruzain	Rhodesain	FheL1	FheL2
SCCA1	1.6	8.6	3.9	36	11
SCCA2	91.7	89	87	66	87
SCCA1 RSL2	104	96	80	nd	64
SCCA2 RSL1	61	46	60	nd	16
SCCA1 mut1(G351V)	8.5	31	5.5	nd	36
SCCA1 mut2(F352V)	33	59	36	nd	23
SCCA1 mut3(G353E)	42	78	30	nd	30
SCCA1 mut4(S354L)	1.1	19	6.9	nd	13
SCCA1 mut5(P356S/ T357P)	2.4	29	5.6	nd	14

nd; not determined.
Residual activities (%) at $I/E = 2$ are depicted. The representative data of two experiments are shown.

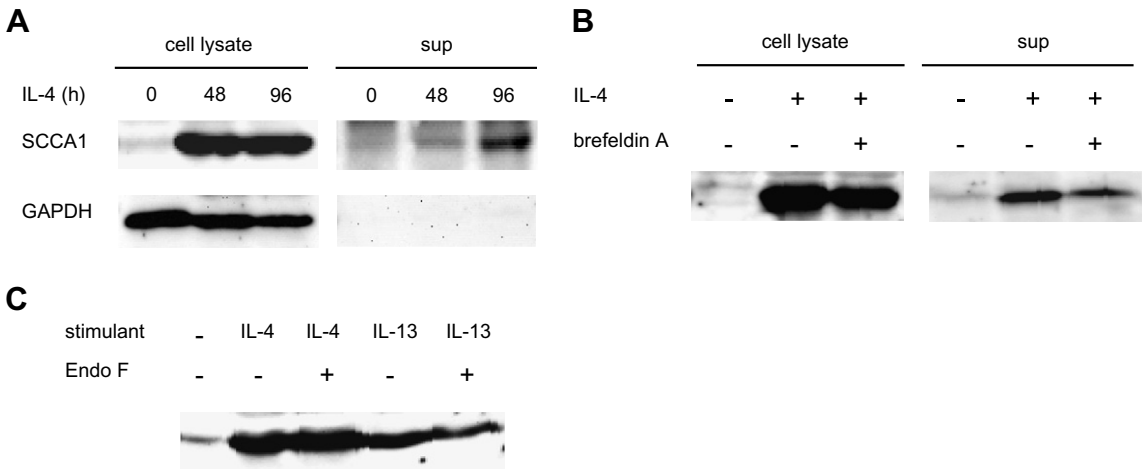

Fig. 3. Secretion of SCCA proteins from human keratinocytes. (A,B) Human keratinocytes were stimulated with 10 ng/ml of IL-4 for the indicated time periods (A) or for 96 h (B) in the presence of 2 μg/ml of brefeldin A. The cell lysates and the supernatants concentrated by trichloroacetic acid (sup) were subjected to SDS–PAGE and blotted with anti-SCCA1 serum (A,B) or anti-GAPDH Ab (A). (C) The cell lysates of human keratinocytes stimulated with 10 ng/ml of IL-4 or 50 ng/ml of IL-13 were treated with 25 U/μl of *N*-glycosidase F (Endo F) and subjected to SDS–PAGE, followed by blotting with anti-SCCA1 serum.

4. Discussion

In this study, we found that SCCA1 had broad-spectrum inhibitory activity on parasite-derived Clan CA, family C1 cysteine proteases, and so might act as a defense mechanism against parasites. To our knowledge, this is the first evidence that a parasite protease can interact with a host protease inhibitor, thereby acting as an anti-pathogen defense mechanism. The notion that SCCA1 is a component of the host defense against parasitic pathogens is supported by two observations. The first is that SCCA1 is inducible by Th2-type cytokines, IL-4 and IL-13, which are known to play critical roles in protecting against helminths [2,3]. The second key observation is that SCCA1 appears to be secreted from keratinocytes via an ER/Golgi-independent pathway, because SCCA proteins were secreted from keratinocytes or HEK293T cells without cleavage of their N-terminal portions, without N-glycosylation, and in a way not blocked by brefeldin A (Fig. 3, data not shown). Since it was reported that IL-1β and galectin-1 were exported from the cells by an ER/Golgi-independent pathway (also known as unconventional protein secretion or nonclassical protein export pathway), the list of proteins demonstrated to be secreted by this pathway is growing [14]. Our present study suggests that SCCA molecules are novel secreted proteins employing an ER/Golgi-independent pathway.

The finding that SCCA1 inhibits several parasite-derived cathepsin L-like proteases in spite of their low homologies (Suppl. Fig. 1) indicates that the elements in the proteases required for the interaction with SCCA1 might be well conserved. It was suggested that SCCA1 inhibited FheCL2, but not FheCL1 (Fig. 1), because of the difference in their substrate specificity in that FheCL2, but not FheCL1, cleaves the substrates having hydrophobic Pro or Val at the P2 position, corresponding to the composition of SCCA1 (Fig. 2B) [15].

We furthermore found that resistance to cleavage by parasite-derived cysteine proteases contributed to the potent inhibitory activities of SCCA1 (Fig. 2A) and that Phe-352 and Gly-353 at the positions of P1′ and P2′ were critical for these effects (Table 1 and Fig. 2B). We previously demonstrated that SCCA2 was resistant to cleavage by Der p 1, whereas SCCA1 was quickly digested by papain [4], indicating that resistance to cleavage by cysteine proteases is a common underlying mechanism of potent inhibitory activities of SCCA proteins.

In conclusion, we found that SCCA1 inhibits the catalytic activities of parasite-derived cysteine proteases, which might act as a defense mechanism against invading parasites.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.07.072.

References

[1] Silverman, G.A. et al. (2001) The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. J. Biol. Chem. 276, 33293–33296.
[2] Yuyama, N. et al. (2002) Analysis of novel disease-related genes in bronchial asthma. Cytokine 19, 287–296.
[3] Mitsuishi, K. et al. (2005) The squamous cell carcinoma antigens as relevant biomarkers of atopic dermatitis. Clin. Exp. Allergy 35, 1327–1333.
[4] Sakata, Y. et al. (2004) The squamous cell carcinoma antigen 2 inhibits the cysteine proteinase activity of a major mite allergen, Der p 1. J. Biol. Chem. 279, 5081–5087.
[5] Sajid, M. and McKerrow, J.H. (2002) Cysteine proteases of parasitic organisms. Mol. Biochem. Parasitol. 120, 1–21.
[6] Finkelman, F.D. and Urban Jr., J.F. (2001) The other side of the coin: the protective role of the TH2 cytokines. J. Allergy Clin. Immunol. 107, 772–780.
[7] Masumoto, K., Sakata, Y., Arima, K., Nakao, I. and Izuhara, K. (2003) Inhibitory mechanism of a cross-class serpin, the squamous cell carcinoma antigen 1. J. Biol. Chem. 278, 45296–45304.
[8] Sakata, Y. et al. (2004) Characterization of novel squamous cell carcinoma antigen-related molecules in mice. Biochem. Biophys. Res. Commun. 324, 1340–1345.
[9] Sanderson, S.J. et al. (2000) Expression and characterization of a recombinant cysteine proteinase of *Leishmania mexicana*. Biochem. J. 347, 383–388.
[10] Eakin, A.E., McGrath, M.E., McKerrow, J.H., Fletterick, R.J. and Craik, C.S. (1993) Production of crystallizable cruzain, the major cysteine protease from *Trypanosoma cruzi*. J. Biol. Chem. 268, 6115–6118.
[11] Caffrey, C.R. et al. (2001) Active site mapping, biochemical properties and subcellular localization of rhodesain, the major cysteine protease of *Trypanosoma brucei rhodesiense*. Mol. Biochem. Parasitol. 118, 61–73.
[12] Collins, P.R. et al. (2004) Cathepsin L1, the major protease involved in liver fluke (*Fasciola hepatica*) virulence: propetide cleavage sites and autoactivation of the zymogen secreted from gastrodermal cells. J. Biol. Chem. 279, 17038–17046.
[13] Takii, R., Kadowaki, T., Baba, A., Tsukuba, T. and Yamamoto, K. (2005) A functional virulence complex composed of gingipains, adhesins, and lipopolysaccharide shows high affinity to host cells and matrix proteins and escapes recognition by host immune systems. Infect Immun. 73, 883–893.
[14] Nickel, W. (2003) The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. Eur. J. Biochem. 270, 2109–2119.
[15] Dowd, A.J., Dalton, J.P., Loukas, A.C., Prociv, P. and Brindley, P.J. (1994) Secretion of cysteine proteinase activity by the zoonotic hookworm *Ancylostoma caninum*. Am. J. Trop. Med. Hyg. 51, 341–347.